Programmed cell death and AIDS: Significance of T-cell apoptosis in pathogenic and nonpathogenic primate lentiviral infections

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ABSTRACT   We have proposed that inappropriate induction of programmed cell death (PCD) or apoptosis, a physiological cell-suicide process, may play a role in the pathogenesis of AIDS. This model has been supported by several reports of abnormal levels of PCD in vitro in both CD4+ and CD8+ T cells from human immunodeficiency virus type 1 (HIV-1)-infected persons. To further assess the significance of such a process in AIDS pathogenesis, in vitro PCD was compared in HIV-1-infected persons and in various primate models that allow discrimination between pathogenic and nonpathogenic chronic lentiviral infection either in the same species, such as rhesus macaques infected with different simian immunodeficiency viruses (SIV), or in different species, such as SIV-infected African green monkeys and HIV-1-infected chimpanzees. Abnormal levels of PCD in CD4+-T-cell-depleted peripheral blood mononuclear cells (PBMC), containing the CD8+ T cells, were observed in both pathogenic and nonpathogenic models. However, abnormal levels of PCD in the CD8+-T-cell-depleted PBMC, containing the CD4+ T cells, was only observed in the two models leading to AIDS: HIV-1-infected persons and rhesus macaques infected with a pathogenic strain of SIV. This suggests that inappropriate T-cell PCD in HIV-1-infected persons involves two distinct processes: one, concerning CD4+ T cells, is closely related to AIDS pathogenesis; and the other, concerning CD8+ T cells, may be a consequence of immune stimulation with no direct link to AIDS pathogenesis.

Human immunodeficiency virus type 1 (HIV-1) infection in humans leads in about 10 years to CD4+ T-cell depletion and AIDS (1). Several years before the onset of CD4+ T-cell depletion, HIV-1-infected persons also show early T-cell functional defects characterized in vivo by a loss of cell-mediated delayed-type hypersensitivity reactions and in vitro by a failure of T cells to proliferate in response to T-cell receptor stimulation by recall antigens and by various mitogens (1–5). We have proposed that both early T-cell dysfunction and late T-cell depletion in HIV-1-infected persons may be related to the inappropriate induction of programmed cell death (PCD) caused by indirect interference of HIV with intercellular signaling (6, 7). Unlike necrosis and cell degeneration, PCD or apoptosis is a physiological cell-suicide process that can be induced or suppressed by activation signals provided by the local environment (8, 9); the involvement of such a process in AIDS pathogenesis could have potential therapeutic implications (6, 7).

Experimental support for our model has been provided by a series of observations from several laboratories, including ours (10–20), showing that (i) in vitro dysfunction of CD4+

and CD8+ T cells from HIV-1-infected persons is related to abnormal induction of PCD (10–14), (ii) the cytopathogenic effect of HIV-1 in CD4+ T cells is due to PCD induction (15, 16), (iii) the crosslinking of CD4 by the HIV-1 envelope protein triggers PCD in uninfected CD4+ T cells (17–19), and (iv) HIV-1 thymus infection leads to thymocyte depletion because of in vivo induction of thymocyte PCD (20). In addition, recent findings have indicated that transient abnormal T-cell PCD can also be observed during acute viral infections that lead to transient immunosuppression (21–23). In this context, an essential question that has remained unresolved for most abnormal features identified so far in HIV-1-infected persons is whether chronic induction of PCD plays a central role in AIDS pathogenesis or is merely a consequence of ongoing and ineffective stimulation of the immune system in a chronic viral infection. To address this question, we have compared in vitro T-cell PCD induction in HIV-1-infected persons and in various primate models that allow one to discriminate between biological features associated with pathogenic and nonpathogenic chronic lentiviral infections.

MATERIAL AND METHODS

Human and Primate Blood Samples. Heparinized venous peripheral blood was obtained at Hôpital Dron (Tourcoing, France) from 24 HIV-1-seropositive adults with CD4+ T-cell counts ranging from 1200 to 60 cells per mm^3 (mean ± SD, 439 ± 376) and 12 HIV-seronegative healthy controls. Heparinized venous peripheral blood was also obtained from various primate species. Chimpanzees, five experimentally infected with the HIV-1-LAI strain (nos. 87, 120, 435, 487, and 527) and three uninfected controls, were housed at the Laboratory for Experimental Medicine and Surgery in Primates (Tuxedo, NY). African green monkeys, two naturally infected by the SIVagm strain (nos. 92017 and 92018) and two uninfected, were housed at Institut Pasteur (Dakar, Senegal). Rhesus macaques (Macaca mulatta) were housed at the primate center, Institut Pasteur (Paris). Ten (nos. P3, P4, OH430, 501, 502, 8718, 49854, 51127, 51179, and 51496) were experimentally infected with the pathogenic SIVmac251 viral strain provided by R. Desrosiers (Harvard Medical School), 4 (nos. 51479, 51182, 51184, and 51192) were experimentally infected with the nonpathogenic SIVmac251 molecular recombinant viral clone BK28 [courtesy of J. Mullins (Stanford University

Medical School)] mutagenized to encode a full-length transmembrane glycoprotein gp41, and 6 were uninfected controls.

Antibodies and Reagents. Murine anti-human monoclonal antibodies (mAb) used were as follows: anti-CD4 (Leu-3a, Becton Dickinson; or OKT4, Ortho Diagnostics), anti-CD8 (Leu-2a; Becton Dickinson), anti-CD14 (IOM2), anti-CD19 (IOB4), anti-CD56 (IOT56), anti-HLA class II antigens (IOT2a; Immunotech, Luminy, France), and anti-CD3 (X35-7 ascites, IgG2a) from D. Bourel (Centre Régional de Transfusion Sanguine, Rennes, France). Other reagents were staphylococcal enterotoxin B (SEB) (Toxin Technology, Madison, WI), pokeweed mitogen (PWM), concanavalin A (Con A), ionomycine, Hoechst 33342 dye (bisBenzimide; Sigma), and acridine orange dye (Immunotech).

Cell Preparation and Culture. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by Ficoll/Hypaque density gradient centrifugation and were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (Boehringer Mannheim), 2 mM L-glutamine, 1 mM sodium pyruvate (GIBCO), and 8 μg of gentamicin (Gentalline; Schering–Plough) per ml. In some experiments, PBMC were depleted of either CD4$^+$ or CD8$^+$ T cells by negative selection with anti-CD4 or anti-CD8 mAb and magnetic beads coated with anti-mouse IgG (Dynal, Biosys, Compiègne, France) as described (10–13); contaminating CD4$^+$

or CD8$^+$ T cells were <5% as assessed by flow cytofluorimetry (flow cytometer: Epics Profile, Coulter). Purified CD4$^+$ T cells (95% pure, as assessed by flow cytofluorimetry) were prepared by the same procedure of negative selection; after PBMC were plated to Petri dishes, nonadherent cells were incubated with anti-CD14, -CD19, -CD56, -CD8, and -HLA class II mAbs and then with the magnetic beads coated with anti-mouse IgG and were removed. Cells were cultured in 96-well culture plates (Falcon, Becton Dickinson) at 2.5×10^5 per ml, or in some experiments at 10^6 per ml or at 2×10^6 per ml, in 24-well plates (Nunc). For T-cell activation, stimuli used were: PWM (10 μg/ml), SEB (1 μg/ml), Con A (5 μg/ml), ionomycin (1 μg/ml), or anti-CD3 mAb ascites at 1:5000 final dilution. PWM, SEB, and Con A induced proliferation in PBMC from uninfected primates of the three explored species.

Measurement of Apoptosis. Apoptosis was measured by four methods. (*i*) Under the light microscope, cells counted as apoptotic included cells with characteristic nuclear chromatin condensation and fragmentation (9) as well as already dead cells that had lost trypan blue exclusion capacity. (*ii*) Under a fluorescent microscope (Leica, Rueil Malmaison, France), nuclear changes were visualized after incubation with Hoechst 33342 nuclear dye (bisBenzimide; 0.5 μg/ml) (24) for 5 min at room temperature. (*iii*) Percentage of apoptotic cells was quantitated by flow cytofluorimetric analysis of 10^6 cells incubated with acridine orange nuclear
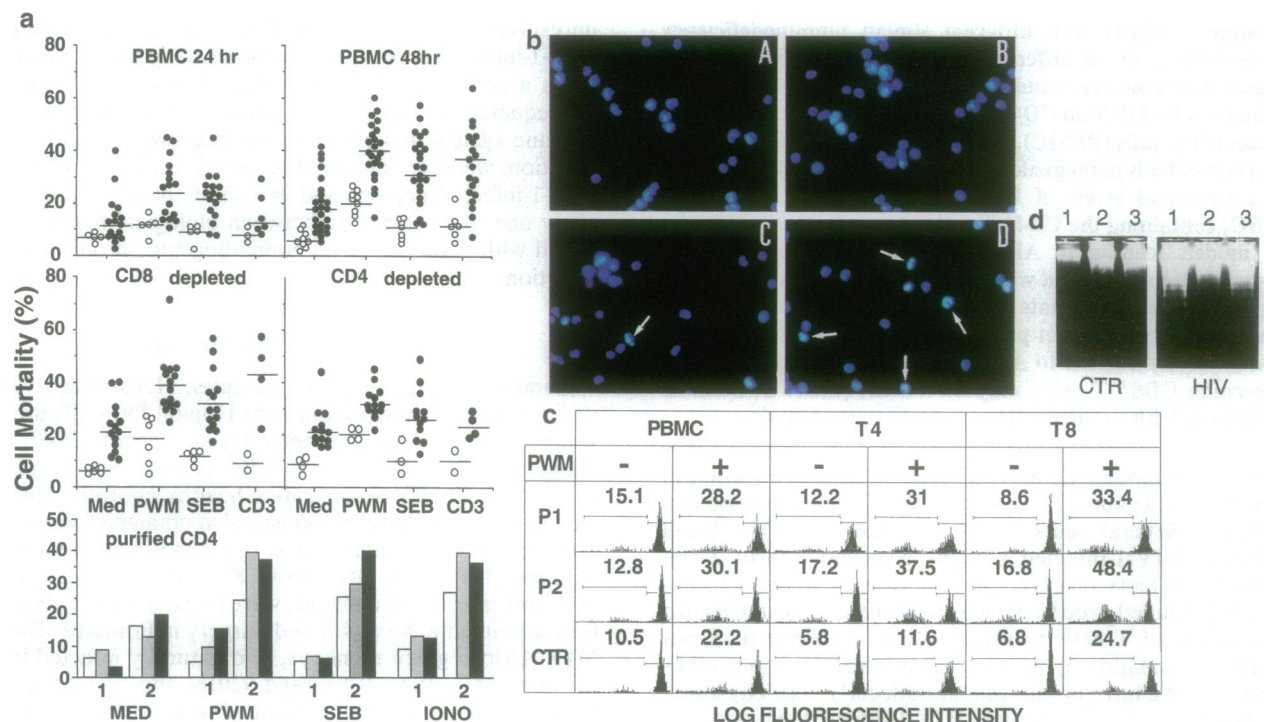

FIG. 1. Apoptosis in PBMC from HIV-1-infected persons. (*a*) Percentage of dead cells. (*a Top*) Percentage of apoptotic cells in PBMC from 24 HIV-1-infected persons (●) and 12 seronegative controls (○) after 24 or 48 hr of culture with medium (MED), PWM, SEB, or anti-CD3 mAb (CD3); apoptosis was assessed under the light microscope; horizontal lines represent the mean value in each group of individuals. (*a Middle*) Similar experiments were carried out after 24 hr of culture in PBMC that had first been depleted in either CD8$^+$ or CD4$^+$ T cells; in each condition (*Top* and *Middle*) percentages of apoptotic cells in HIV-1-infected persons were significantly higher than in seronegative controls ($P < 0.001$). (*a Bottom*) Percentage of apoptotic cells in PBMC (□), purified CD4$^+$ T cells (□), and purified CD4$^+$ T cells plus purified monocytes (1:1 ratio) (■) from one control (columns labeled 1) and one HIV-infected person (columns 2), after a 24-hr culture with medium, PWM, SEB, or ionomycin (IONO). (*b*) Fluorescent microscopic analysis. PBMC from one human control (*A* and *B*) and one HIV-1-infected person (*C* and *D*) were cultured for 24 hr in the absence (*A* and *C*) or presence (*B* and *D*) of PWM and examined under a fluorescent microscope. Arrows indicate typical nuclear chromatin condensation or fragmentation in apoptotic cells in *C* and *D*. (Hoechst 33342; ×400.) (*c*) Flow cytofluorimetric analysis. PBMC and CD8$^+$- or CD4$^+$-T-cell-depleted PBMC (T4 and T8, respectively) from one control (CTR) and two HIV-1-infected persons (P1 and P2) were cultured for 24 hr with medium (−) or PWM (+), and were analyzed by flow cytometry after acridine orange staining. The percentages of apoptotic cells corresponding to the peaks of reduced fluorescence intensity are indicated in each experimental condition. (*d*) DNA fragmentation. Agarose gel electrophoresis of DNA extracted from unfractionated PBMC (lanes 1), from CD8$^+$-T-cell-depleted PBMC (lanes 2), or from CD4$^+$-T-cell-depleted PBMC (lanes 3) from one HIV-infected person (*Right*) and one healthy control (CTR) (*Left*) after 24 hr of culture with PWM.

dye (0.1 µg/ml) for 5 min; lymphocytes were gated under forward- and side-light scatter, with apoptotic cells representing a characteristic distinct peak of reduced fluorescence intensity and forward scatter below the peak of living cells (25). (*iv*) Qualitative analysis of DNA fragmentation was performed by agarose gel electrophoresis of DNA extracted from 2×10^6 cells (10–13). Specificity of these techniques was validated by using normal human thymocytes treated *in vitro* with dexamethasone, a known inducer of apoptosis (9); cell sorting after flow cytofluorimetric analysis (Epics Elite, Coulter) showed that the four features of apoptosis explored were present in the sorted apoptotic thymocyte population and none in the sorted living cells (F.D.B., unpublished data).

Statistical Analysis. Statistical significance was assessed by Student's *t* test.

RESULTS

Pathogenic Models of Chronic Lentiviral Infection. *HIV-1-infected humans.* PBMC from HIV-seronegative controls cultured in medium alone showed low levels of spontaneous apoptosis that were enhanced by *in vitro* stimulation with PWM. Spontaneous apoptosis was significantly higher in PBMC from HIV-1-infected persons and was markedly enhanced by *in vitro* stimulation with PWM, SEB superantigen, or the CD3 mAb (Fig. 1a). Abnormal levels of PCD were also observed in CD8$^+$-T-cell-depleted PBMC, containing the CD4$^+$ T-cell population; in CD4$^+$-T-cell-depleted PBMC, containing the CD8$^+$ T-cell population; and in 95% pure CD4$^+$ T cells in either the absence or the presence of added syngeneic adherent accessory cells (Fig. 1a).

Apoptosis was further assessed by three different methods (see *Material and Methods*), including fluorescence microscopy after staining with the nuclear dye Hoechst 33342 (Fig. 1b),

flow cytofluorimetric analysis after staining with the nuclear dye acridine orange (Fig. 1c), and DNA fragmentation by the DNA electrophoresis technique (Fig. 1d). Percentages of apoptotic cells were similar when assessed under the light microscope (Fig. 1a) and by flow cytofluorimetric analysis (Fig. 1c).

Rhesus macaques infected with the SIVmac251 strain. Experimental infection of rhesus macaques with a number of SIV strains, including SIVmac251, results in 1–3 years in an AIDS-like disease remarkably similar to that observed in HIV-1-infected humans (26–28). Ten SIVmac251-infected rhesus macaques and 6 uninfected controls were explored. A first series of 7 macaques was infected for 9–22 months, 6 being explored two consecutive times with a 1-month interval. Enhanced levels of apoptosis were observed in PBMC and in CD8$^+$- or CD4$^+$-T-cell-depleted PBMC from 2 infected macaques, at both time points, and in 1 additional macaque at one time point only (Fig. 2a). As for the HIV-1-infected persons (Fig. 1 *b–d*), apoptosis in the macaques was further assessed by three different methods (Fig. 2 *b–d*). Abnormal PCD levels in these 3 macaques were not correlated with the inoculate median animal infectious dose (aID$_{50}$) [10 aID$_{50}$ in 1 macaque, 3000 tissue culture median infectious dose (TCID$_{50}$) corresponding to 3000 aID$_{50}$ (ref. 29) in the other 2], with the duration of infection (9, 11, and 22 months) or with the CD4$^+$ T-cell counts at the time of the study (550 per mm^3 in one, 1200 per mm^3 in the other 2).

Of the 10 infected macaques studied, another series of 3 macaques was infected (with 10 aID$_{50}$ of the same SIVmac251 strain) for only 2 months at the onset of the study and were explored four consecutive times over a period of 3 months. Abnormal levels of *in vitro* PCD were observed in PBMC and in CD8$^+$- or CD4$^+$-T-cell-depleted PBMC from 2 of the 3 macaques (Fig. 3a).
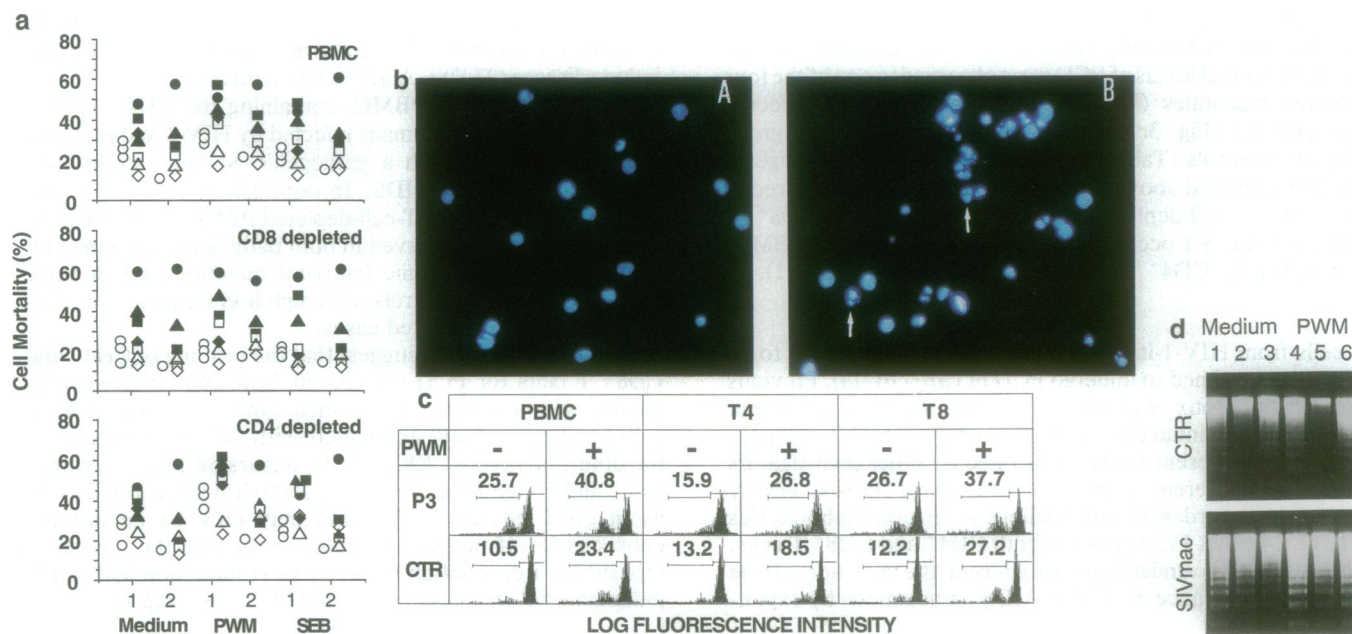

FIG. 2.   Apoptosis in PBMC from macaques infected with a pathogenic strain of SIV. (*a*) Percentage of dead cells. Percentage of apoptotic cells was assessed as in Fig. 1a in PBMC and CD8$^+$- or CD4$^+$-T-cell-depleted PBMC from seven macaques experimentally infected with SIV for 9–22 months and from three uninfected controls. Experiments were carried out twice (columns 1 and 2) with a 1-month interval, the first experiment being performed after 48 hr of culture with medium, PWM, or SEB and the second after only 24 hr of culture. All symbols represent infected macaques except ○, which represents controls. (*b*) Fluorescent microscopic analysis. PBMC from one macaque control (A) and one SIV-infected macaque (B) were cultured for 24 hr with PWM and then treated with Hoechst 33342 as in Fig. 1b. Arrows show typical nuclear chromatin condensation or fragmentation in some of the numerous apoptotic cells in *b*. (×400.) (*c*) Flow cytofluorimetric analysis. PBMC and CD8$^+$- or CD4$^+$-T-cell-depleted PBMC (T4 and T8, respectively) from one macaque control (CTR) and one SIV-infected macaque (P3) were cultured for 24 hr with medium (−) or PWM (+) and analyzed by flow cytometry as in Fig. 1c. The percentage of apoptotic cells is indicated in each experimental condition. (*d*) DNA fragmentation. Agarose gel electrophoresis of DNA extracted from unfractioned PBMC (lanes 1 and 4), CD8$^+$-T-cell-depleted PBMC (lanes 2 and 5), or CD4$^+$-T-cell-depleted PBMC (lanes 3 and 6) from the SIV-infected macaque (SIVmac) and the control (CTR) shown in *b* after 24 hr of culture with medium (lanes 1, 2, and 3) or PWM (lanes 4, 5, and 6).
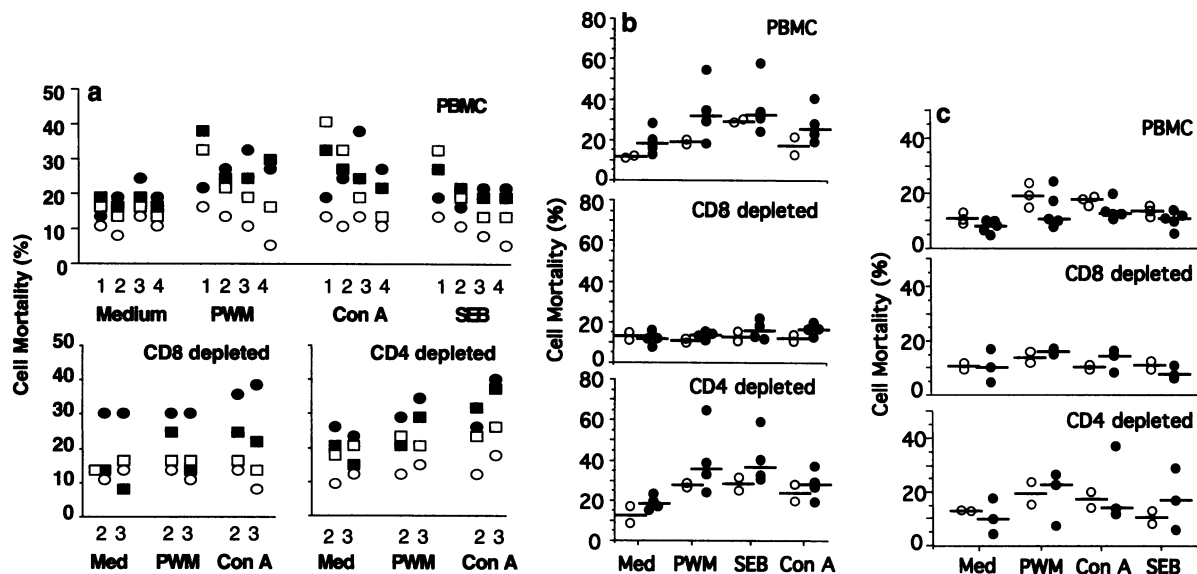
FIG. 3.   Apoptosis in PBMC from pathogenic and nonpathogenic primate models of lentiviral infection. The percentage of apoptotic cells was assessed as in Fig. 1a and Fig. 2a in PBMC and CD8⁺- or CD4⁺-T-cell-depleted PBMC after 24 hr of culture with medium (med), PWM, Con A, or SEB. (a) Macaques infected with a pathogenic strain of SIV. Experiments 1–4 were carried out during a 3-month period beginning 2 months after experimental infection of the three macaques. All symbols represent infected macaques, except "○," which represents the uninfected control (three different controls were used for experiments 1–4). (b) Macaques infected with a nonpathogenic molecular clone of SIV. Symbols represent four infected (●) and two uninfected (○) macaques. Horizontal lines represent the mean values. (c) HIV-infected chimpanzees. Symbols represent five HIV-1-infected (●) and three uninfected (○) chimpanzees. Horizontal lines represent the mean values.

Nonpathogenic Models of Chronic Lentiviral Infection. Primate models explored included four rhesus macaques experimentally infected for 11 months with 3.20 to 320 aID₅₀ of the nonpathogenic viral molecular clone SIVmac251 (27, 28) and two controls; five chimpanzees experimentally infected with HIV-1 for up to 5 years and three controls; and two African green monkees naturally infected with SIVagm and two controls. Abnormal levels of PCD were observed in one of the four infected macaques (Fig. 3b), in one of the five infected chimpanzees (Fig. 3c) and in two uninfected African green monkey controls (Table 1). In contrast with the pathogenic models described above, PCD in these cases only occurred in the CD4⁺-T-cell-depleted PBMC, containing the CD8⁺ T cells, and did not occur in the CD8⁺-T-cell-depleted PBMC, containing the CD4⁺ T cells (Fig. 3 b and c and Table 1).

DISCUSSION

T cells from HIV-1-infected persons have been shown to be abnormally primed to undergo PCD *in vitro* (10–14). Previous findings by Groux *et al.* (10) in our laboratory indicated that abnormal PCD induction only involved the CD4⁺ T-cell population. The present study, performed by other investigators using four different assays to measure apoptosis, clearly shows, in accordance with findings from other laboratories (11, 12, 14), that PCD involves both CD4⁺ and CD8⁺ T cells. This study was undertaken to address the question of the possible significance of PCD in AIDS pathogenesis by exploring primate models of pathogenic or nonpathogenic chronic lentiviral infection. These included chimpanzees experimentally infected with HIV-1 that do not develop disease (30); African green monkeys naturally infected with SIVagm that do not develop disease (31); and rhesus macaques experimentally infected either with a viral strain of SIVmac that induces AIDS (26–28) or with a molecular viral clone of SIVmac that does not induce disease (27, 28). Abnormal *in vitro* induction of PCD in CD8⁺-T-cell-depleted PBMC, containing the CD4⁺ T cells, was only observed in humans infected by HIV-1 and in rhesus macaques infected with a pathogenic SIVmac strain—two situations leading to AIDS. In contrast, enhanced *in vitro* levels of PCD in CD4⁺-T-cell-depleted PBMC, containing the CD8⁺ T cells, was observed in both pathogenic and nonpathogenic models of chronic lentiviral infection and in some uninfected primate controls, although less frequently in nonpathogenic and uninfected cases.

Together, these data suggest that the priming of CD4⁺ and CD8⁺ T cells for PCD may result from two different processes, with a distinct significance. One, involving CD4⁺ T cells, appears closely associated with AIDS pathogenesis; the other, involving CD8⁺ T cells, occurs during both pathogenic and nonpathogenic chronic lentiviral infection, as·well as in other circumstances, and may only be an indirect consequence of immune stimulation. During the course of this study, a correlation between PCD induction and AIDS pathogenesis was also suggested by other reports of (i)

Table 1.   Apoptosis in PBMC from SIVagm-infected and from uninfected African green monkeys

| | Controls | | | | SIVagm-infected | | | | | |
| | 93001 | 92016 | | | 92017 | | | 92018 | | |
	PBMC	PBMC	T4	T8	PBMC	T4	T8	PBMC	T4	T8
Medium	11	5.7	10.8	5.3	4.4	3.3	4.2	8.2	9.8	3.2
PWM	25.8	21.1	14	22.2	11.6	10.3	14.2	8.3	7.9	9.8
Con A	25.6	28.4	13.3	33.1	11.5	8.3	11.7	8.8	6.7	14.7
SEB	11.1	12.9	10.5	8.9	7.9	7.7	9.7	8.3	6.9	10.7

PBMC or CD8⁺-T-cell-depleted PBMC (T4) or CD4⁺-T-cell-depleted PBMC (T8) were cultured for 24 hr with medium, PWM, Con A, or SEB. Values represent the percentage of apoptotic cells.

abnormal *in vitro* PCD induction in PBMC from rhesus macaques infected with a pathogenic strain of SIV (12, 32) and from cats infected with a pathogenic feline immunodeficiency virus (33), and (*ii*) a lack of PCD induction in PBMC from HIV-1-infected chimpanzees (12, 34). The absence of comparison between pathogenic and nonpathogenic infections in the same species and the absence of exploration of PCD in the CD4$^+$ or CD8$^+$ T-cell populations may explain the clear-cut conclusion of these studies that abnormal PCD induction in unfractionated PBMC strictly correlates with pathogenic lentiviral infection (12, 32–34).

Our observation that both CD4$^+$ and CD8$^+$ T cells undergo inappropriate *in vitro* PCD in pathogenic models of lentiviral infection, whereas selective CD4$^+$ T-cell depletion is an *in vivo* feature of progression to AIDS, raises the question of the significance of these findings. A first possibility, that *in vitro* T-cell PCD does not reflect the *in vivo* fate of the T cells, seems unlikely in view of recent preliminary findings of high levels of PCD involving both CD4$^+$ and CD8$^+$ T cells in lymph nodes from HIV-1-infected persons (35). Another possibility is that both CD4$^+$ and CD8$^+$ T cells undergo continuous PCD *in vivo* during pathogenic lentiviral infections but that renewal of CD4$^+$ T cells in such a situation is selectively impaired. A preferential capacity of CD8$^+$ T cells to renew or to expand in the periphery is suggested by the recent observation that whole-body irradiation in SIV-infected macaques with normal CD4$^+$ T-cell counts induces a profound depletion of both CD4$^+$ and CD8$^+$ T cells that is followed by a rapid reappearance of CD8$^+$ T cells, but by a prolonged state of CD4$^+$ T-cell depletion (36). An additional effect of pathogenic lentiviruses on the renewal capacity of CD4$^+$ T cells is suggested by the finding that HIV-1 infection of severe combined immunodeficient SCIDhu mice reconstituted with human fetal thymuses induces a profound thymocyte depletion that selectively affects CD4$^+$ thymocytes (20).

Our study suggests that abnormal CD4$^+$ T-cell PCD is specific for pathogenic lentiviral infection but is not detected in each case. It was observed in most but not all of 24 HIV-1-infected persons and in 5 of the 10 macaques infected with the pathogenic strain of SIV, but not at all time points, suggesting a fluctuation of this process during the course of the infection. T cells that are recirculating in the peripheral blood represent at any given time <2% of the total T lymphocyte pool in the body (37). It is therefore possible that the presence of T cells primed for PCD in the peripheral blood only represents an intermittent and indirect consequence of a continuous process of T-cell PCD induction that would mainly occur in the lymphoid organs, in which most of the T cells and most of the viral burden are located (1). Histopathological studies of T-cell PCD (38) in the lymphoid organs will be required to further assess the *in vivo* relevance of our findings in the pathogenic and nonpathogenic primate models of lentiviral infection that we have explored.

In conclusion, the present study suggests a mechanism for the immunopathogenesis of AIDS by establishing a correlation between the abnormal priming of CD4$^+$ T cells for PCD and the pathogenic nature of a lentiviral infection. It also indicates that rhesus macaques infected with pathogenic strains of SIV show biological features closely resembling those of HIV-1-infected individuals and therefore represent an appropriate model to test the potential beneficial effect on AIDS progression of therapeutic strategies aimed at early prevention of PCD.

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